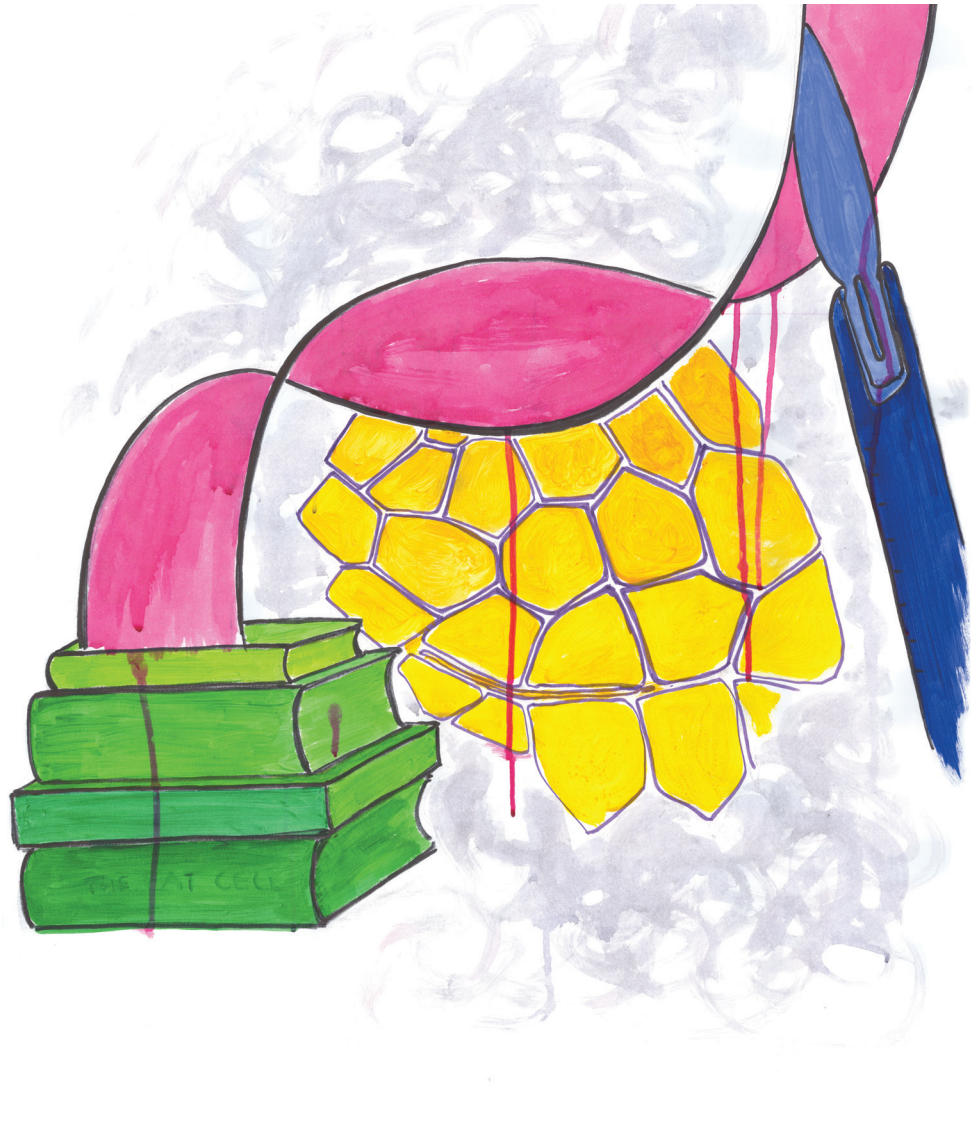

CHAPTER 6

Platelet-Rich Plasma (PRP) influences expansion and paracrine function of adipose-derived stromal cells (ADSC) in a dose-dependent fashion

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ABSTRACT

Background

Lipofilling is a treatment modality to restore tissue volume. Both platelet-rich plasma and adipose-derived stromal cells have been reported to augment the efficacy of lipofilling, yet results are not conclusive. The authors hypothesized that the variation reported in literature is caused by a dose-dependent influence of platelet-rich plasma on adipose-derived stromal cells.

Methods

Whole blood (n = 3) was used to generate platelet-rich plasma, which was diluted with Dulbecco's Modified Eagle Medium to 15%, 5%, and 1.7%, with 15% platelet-poor plasma and 10% fetal calf serum as controls. Pooled adipose-derived stromal cells (n = 3) were cultured in these media. Gene expression was assessed, along with angiogenic sprouting of endothelial cells by conditioned medium and platelet-rich plasma.

Results

platelet-rich plasma in culture medium affected the expression of genes in a dose-dependent manner. The 15% concentration stimulated proliferation almost eightfold. Mesenchymal markers were unaffected. Interestingly, expression of collagens type 1 and 3 increased at lower concentrations, whereas transforming growth factor-β showed reduced expression in lower concentrations. Proangiogenic gene expression was unaltered or strongly reduced in a dose-dependent manner. platelet-rich plasma promoted endothelial sprouting and survival in a dose-dependent manner; however, conditioned medium from adipose-derived stromal cells exposed to platelet-rich plasma blocked endothelial sprouting capabilities.

Conclusion

The dose-dependent influence of platelet-rich plasma on the therapeutic capacity of adipose-derived stromal cells conditioned medium in vitro warrants caution in clinical trials.

INTRODUCTION

Fat-grafting, or lipofilling has become an accepted treatment modality in the (re)construction of tissue volume. Indications vary from acquired[1] to hereditary loss[2] of volume in both aesthetic as well as reconstructive surgery. Although technical improvements have increased the overall fat graft take dramatically[3], the level of fat graft survival is still uncertain and suboptimal[4]. Various methods have been suggested to increase graft take, which include negative pressure garments[5] (BRAVA), addition of ADSC to grafts[6] and addition of platelet derived growth factors[7] or platelet rich plasma (PRP). The efficacy of these procedures however, still has to be proven in randomized clinical trials.

Besides the volumetric effect observed after a lipofilling procedure, skin rejuvenation features are observed like decreased pore size, improved elasticity and suppression of inflammatory skin conditions as well as reduction of existing scars[1,8]. The presence of Adipose-Derived Stromal Cells (ADSC) in the lipograft is suggested to participate in tissue rejuvenation features as well as improved would healing[9]. These ADSC achieve this either by direct support of the lipograft during the first days, facilitating vessel ingrowth, or by differentiation into adipocytes or both[10].

PRP, as investigated by Marx *et al.*[11], is rich in growth factors that support wound healing in normal physiology. These growth factors, such as PDGF, TGFβ and VEGF locally influence migration, proliferation and differentiation[12] of several cell-types including endothelial cells (angiogenesis) and (myo)fibroblasts (deposition of extracellular matrix (ECM)). In animal studies, PRP augments graft take[13-16], most likely due to improved vascularization. Moreover, addition of PRP to fat grafts, reduces the occurrence of oil cysts[17]. Oil cysts are cytotoxic and hence suppress tissue repair and graft take. However, the clinical efficacy of the use of PRP is highly variable[7,18,19], which causes doubt about the benefit of PRP to support and improve graft take[20].

From a pharmacological point of view, it is to be expected that PRP would act in a dose-dependent fashion in combination with lipofilling. Therefore, the observed clinical variation in the effect of PRP could be due to concentration differences. *In vitro*, fibroblasts and osteoblasts react in a dose-related response to PRP. At low concentrations PRP promotes their proliferation, while at higher concentrations PRP inhibits cell proliferation and migration[21-25].

Studies that explore the influence of PRP on the proliferation and cellular function of ADSC are limited[20]. Besides PRP, ADSC appear a promising factor to influence graft take and tissue regeneration[10,26,27], which underlies our *in vitro* study to investigate the influence of PRP concentration - on the proliferation, phenotype and function of ADSC.

MATERIALS AND METHODS

ADSC isolation and culture

Human adipose tissue was collected from the resected abdominal skin flap from three healthy patients that underwent abdominoplasty for aesthetic reasons. The skin flaps were retrieved after informed consent from the patients. The donors were healthy with a BMI below 30. The use of resected skin flaps as source of ADSC was approved by of the local Ethics Committee of University Medical Centre Groningen given the fact that it was considered the use of anonymized waste material. The skin flaps were processed using a working protocol for ADSC isolation[28]. Isolated ADSC were expanded to passage 3 (P3) in Dulbecco's modified Eagle's medium (DMEM, Lonza) supplemented with 10% fetal bovine serum (FBS) (Thermo Scientific, Hempstead,UK), 1% L-glutamine (Lonza Biowhittaker Verviers,Belgium), and 1% penicillin/ streptomycin (Gibco, Invitrogen, Carlsbad,CA) at 37ºC in a humidified incubator with 5% CO¬2. For the experiments, ADSC of all three donors (P3 and higher) were used as a pool.

Preparation of PRP and PPP

Whole blood was drawn (27 ml) from three healthy volunteers and mixed with 3 ml of citrate (standard ACD-A solution) to prevent clotting. Additional blood was drawn for laboratory analysis of the donor's platelet, RBC and WBC counts. The whole blood citrate mixture was introduced into the Biomet GPS-III device (Biomet Warsaw, Indiana) following the manufacturer's instructions. Fifteen minutes of centrifugation at 2,200xg allowed for separation of the whole blood into its three fractions: erythrocytes, platelet-poor plasma (PPP), and platelet-rich plasma (PRP). Output volume of the PRP was 3mL, which was a ninefold reduction of the input volume of whole blood. PPP and the PRP were collected separately with a syringe through the ports on the GPS-III device. Residual leukocytes and erythrocytes were removed from the PRP fraction by centrifugation at 300x g for 10min[29]. This was essential because both cell types inhibited the proliferation of ADSC (data not shown).

PRP dilution

The PRP was diluted with DMEM to final concentrations of respectively 15%, 5% and 1.7%, while PPP was diluted to 15% only. Alternatively, for experiments with endothelial cells, DMEM was replaced with endothelial cell culture medium (ECM), comprising of RPMI-1640 supplemented with 5mg/ml endothelial cell growth factors[30] (Bovine brain extract), 5U/mL heparin (Leo Pharma, Holland), 100U/mL penicillin, 100mg/mL streptomycin, 2mM L-glutamine. The diluted PRP and PPP were brought to 100U/mL penicillin, 100mg/mL streptomycin and 2 mM L-glutamine too. Upon dilution the PRP was activated through vigorously shaking which resulted in the formation of a clot (Fig. 1). The clot was resuspended by breaking it with a pipet tip, and pipetting it up and down several times. As control, 10% or 20% fetal calf serum (FCS) was used instead of PRP or PPP.

Proliferation

Confluent cultures of pooled ADSC (P4) were seeded in 24 well plates at $4x10^4/cm^2$ (t= -1d). After 24h culture time on DMEM FCS 10% (t=0d), media were replaced by the PRP-PPP conditions that were generated on d0 too. Cells were cultured for 4 days. After 4 days cells were fixed with 2% paraformaldehyde in PBS for 20min. Following extensive washing, cells were permeabilized with 0.5% Triton X-100 in PBS (Sigma-Aldrich, MA). Subsequently, plates were incubated with polyclonal rabbit-anti-human Ki67 antibody (Monosan, Holland) diluted 1:250 in PBS with 10% horse serum (First Link Ltd, UK) for 90 min. Control wells received only detection antibody. After extensive washing, the wells were incubated with donkey-anti-rabbit serum conjugated to Alexa Fluor 488 (Life Technologies, Carlsbad, CA) diluted 1:300 in 0.5µg/mL DAPI PBS and 2% horse serum for 30min followed by a final extensive washes with PBS.

The entire surface of each well was scanned using automated immunofluorescent microscopy (TissueFAXS, TissueGnostics, Vienna, Austria) using a Zeiss AxioObserver Z1 microscope on 10x magnification. Two wells were scanned per condition per PRP donor (n=3). TissueQuest cell analysis software (v4.0.1.0.127) determined the total cell-count and percentage of Ki67 expressing cells.

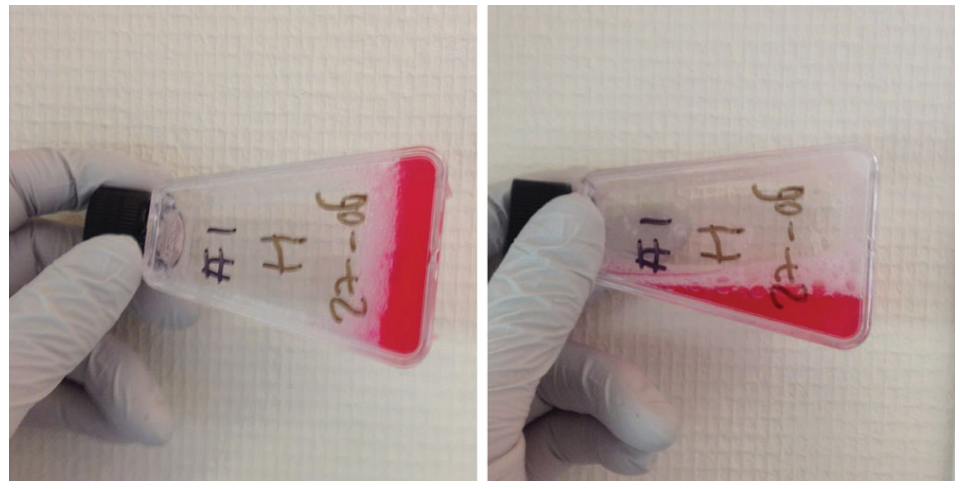

Figure 1 | Activation, gelling and resuspension of PRP. The PRP was diluted with DMEM to final concentrations of respectively 15%, 5% and 1.7%, while PPP was diluted to 15% only.

Left panel: PRP was activated separately after their diluting, through vigorously shaking the tubes, which resulted in the formation of a clot.

Right panel: resuspension of the clot by pipetting up and down several times.

Gene expression by qRT-PCR

At t = -1d confluent cultures of pooled ADSC (P4) were seeded in 6 well plates at $4x10^4/cm^2$. After 24h culture time on DMEM FCS 10% (t=0d), media were again replaced by the medium with 1.7,

5 and 15% PRP respectively or medium with 15% PPP and left in culture for 4d. DMEM with 10% FCS served as control.

Cells were lysed in using Trizol Reagent (Life technologies, Carlsbad, CA) and total RNA was isolated according to the manufacturer's protocol. Two µg of total RNA was reverse transcribed using a First Strand cDNA synthesis kit (Thermo-Scientific, Waltham, MA). The cDNA equivalent of 10ng RNA was used for amplification in 384-well microtiter plates in a final reaction volume of 10 µl containing 6 µM primer mix (forward and reverse) in 5 µl SyberGreen (Life Technologies).

6 µM of human β-actin primer mix was used as a reference gene. PCRs were performed by a ViiA-7 real-time PCR system (Life Technologies), with Cycle threshold (CT) values for individual reactions obtained from the ViiA-7 processing software. Relative expression was calculated using the delta CT method. All reactions were performed in triplicate. Primer sequences for qRT-PCR are listed in Table 1.

Endothelial sprouting assay

Conditioned medium (CM) from pooled ADSC (P4) was prepared by a 4 days pre-culture in ECM with PRP and PPP concentrations as described above, and with 10% FCS as positive control. This was followed by incubation in the absence of PRP or FCS for 16h.

Inner wells of µ-Slide Angiogenesis plates (Ibidi GmbH, Germany) were coated with 10µL MatriGel™ (BD Biosciences, CA) and incubated at 37°C for 2h. Pooled P4 Human Umbilical Vein Endothelial cells (HUVEC, Lonza Biowhittaker) were suspended (200,000 cells/mL) in the prepared conditioned media, ECM-PRP media and ECM-PPP medium using the same dilutions as described above. ECM FCS 20% and CM from ADSC cultured on ECM FCS 10% served as positive control, serumfree ECM as negative. In total, 50µl HUVEC (10,000 cells) was pipetted in every well in triplicate. Plates were incubated after which 2.5x light microscopy images were obtained from every well after 6, 16, 24 and 72h (72h ECM-PRP wells only). The number of sprouts, quantified in number of loops and branches as well as their length, was calculated with the Angiogenesis-analyzer plugin[31] for Image J (National Institutes of Health, Bethesda, Maryland, USA).

Statistics

Data was analyzed by GraphPad Prism (v6.0 OSX, GraphPad Software Inc., LaJolla, CA), and is presented as means with corresponding SEM (±). Statistical significance was determined using one-way ANOVA with Bonferroni's comparison post-hoc analysis. Differences with a calculated change value of $p<0.05$ were considered significant.

Table 1 | Primer sequences for quantitative reverse-transcriptase polymerase chain reaction

Official GENE Symbol	GENE Alias	Sequence Forward	Sequence Reverse
ACTB	beta-Actin	CCAACCGCGAGAAGATGA	CCAGAGGCGTACAGGGATAG
GAPDH	G3PD, Glyceraldehyde-3-Phosphate Dehydrogenase	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC
TGFB1	Transforming Growth Factor Beta 1	ACTACTACGCCAAGGAGGTCAC	TGCTTGAACTTGTCATAGATTTCG
TGFB2	Transforming Growth Factor Beta 2	ATAAATTTCAGCCCAGGTCA	CCAAAAGGGAAGAGATGAAA
TGFB3	Transforming Growth Factor Beta 3	ACACTTGGTTAGACGCCTTC	ACCAAATGAACACAGGGTCT
TAGLN	Transgelin, SM22a	CTGAGGACTATGGGGTCATC	TAGTGCCCATCATTCTTGGT
CNN1	Calponin-1, basic-Calponin, SMCC	CCAACCATACACAGGTGCAG	TCACCTTGTTTCCTTTCGTCTT
PECAM1	CD31, endoCAM, GPIIA	GCAACACAGTCCAGATAGTCGT	GACCTCAAACTGGGCATCAT
VWF	Von Willebrand Factor, Factor 8 (F8)	AGTGAGCCTCTCCGTGTATC	TCACCGGACAGCTTGTAGTA
PDGFB	Platelet-Derived Growth Factor B	CTGCATTTTCCTCTTGTCCT	TTCTGCCCTAGAGAGGAGTG
PDGFRB	Platelet-Derived Growth Factor Receptor Beta, CD140b	CCCTTATCATCCTCATCATGC	CCTTCCATCGGATCTCGTAA
ANGPT1	ANG1, Angiopoietin 1	CTACTGGGCCTCCTCTCATA	TCTCAAATGGAGGAAACCAT
ANGPT2	ANG2, Angiopoietin 2	CAGTTCTTCAGAAGCAGC	TTCAGCACAGTCTCTGAA
VEGFA	Vascular Endothelial Growth Factor A, Vascular Permeability Factor	CCTGAAATGAAGGAAGAGGA	AAATAAAATGGCGAATCCAA
FGF1	Fibroblast Growth Factor 1, acidic FGF	ACCAAGTGGATTCTGCTTCC	CTTGTGGCGCTTTCAAGACT
FGF2	Fibroblast Growth Factor 2, basic FGF	CTGTACCCATACAGCAGCAG	CGCCTAAAGCCATATTCATT
COL1A1	COL1, Collagen Type 1 alpha 1	GGGATTCCCTGGACCTAAAG	GGAACACCTCGCTCTCCA
COL3A1	COL3, Collagen Type 3 alpha 1	CTGGACCCCAGGGTCTTC	CATCTGATCCAGGGTTTCCA
MMP1	Matrix Metalloproteinase-1, Collagenase (CLGN)	GCTAACCTTTGATGCTATAACTACGA	TTTGTGCGCATGTAGAATCTG
MMP2	Matrix Metalloproteinase-2, Gelatinase A (GLNA)	GTTCCCCTTCTTGTTCAATG	CTTGCCATCCTTCTCAAAGT
MMP9	Matrix Metalloproteinase-9, Gelatinase B (GLNB)	GACGATGACGAGTTGTGGT	GAAGATGAAGGGGAAGTGG
HGF	Scatter factor, Hepatocyte Growth Factor	GTTTCCCAGCTGGTATATGG	GGTCTTTTCAGGAATTGTGC
CSPG4	Chondroitin Sulfate Proteoglycan 4, NG2	GAGAGGCAGCTGAGATCAGAA	TGAGAATACGATGTCTGCAGGT
IGF1	Insulin-like Growth Factor 1, Somatomedin-C	ACTCGGGCTGTTTGTTTTAC	GTGTGCTTCTTGACGACTTG
IL1B	Interleukin 1 beta,	GGTTGAGTTTAAGCCAATCCA	TGCTGACCTAGGCTTGATGA
IL6	Interleukin 6	AGCTCAATAAGAAGGGGCCTA	TGAGAAACCCTGGCTTAAGTAGA
IL8	Interleukin 8, Granulocyte Chemotactic Protein 1 (GCP1), CXCL8, TNFAIP1	CTTTCAGAGACAGCAGAGCA	ACAC=AGAGCTGCAGAAATCA
TIMP1	Tissue Inhibitor of Metalloproteinases 1	CCAGCGTTATGAGATCAAGA	AGTATCCGCAGACACTCTCC
TIMP2	Tissue Inhibitor Of Metalloproteinases 2	GAAGAGCCTGAACCACAGGT	CGGGGAGGAGATGTAGCAC
FN1	Fibronectin, GFND2	TCAACTCACAGCTTCTCCAA	TTGATCCCAAACCAAATCTT
LAMA1	Laminin Alpha 1	ATGGAAAATGGCACACTCTT	AGACTGGGTGTGTGGACTTT

RESULTS

PRP donors

A total of 3mL PRP was generated per donor; with donor platelet counts all within normal range (251 ± 39.13, range 180-315). Donors' RBC and WBC counts were normal.

PRP promotes cell proliferation in a dose-dependent fashion

Human ADSC proliferated irrespective of medium supplements PRP or the controls PPP or FCS. Irrespective of the supplements ADSC had a fibroblast-like appearance as observed by microscopy (Fig. 2A), while no visual signs of apoptosis such as blebbing were observed. Moreover, both 5% and 15% PRP had visibly higher cell numbers after 4 days of culture (Fig 2A). This dose-dependent influence of PRP on proliferation of ADSC was corroborated by quantification (Fig. 2B). Total cell numbers were increased in media with 15% PRP ($p<0.05$), while in media with 5% and 15% PRP also more proliferating cells were present as observed by increased expression of Ki-67 (Fig. 2B). In contrast, proliferation was hampered in media with 1.7% PRP, proliferation in media with 15% PPP and 10% FCS were comparable.

Gene expression in ADSC is influenced by PRP in a dose-dependent fashion

The beneficial function of ADSC relies on the expression of genes that influence relevant processes, which include angiogenesis, inflammation and remodeling of the extracellular matrix. The expression of representative genes from each these functions showed a dose-dependent expression to PRP as compared to controls (PPP and FCS, Fig. 3).

Mesenchymal differentiation.

All PRP media induced minor changes in *TAGLN* expression when compared to 10% FCS ($p>0.05$) and to 15% PPP ($p>0.05$). Relative gene expression of *CCN1* was not affected (Fig. 3A). On the other hand the pericytic nature of ADSC did not change too, at least in terms of expression of *PDGFR* and *NG2* (Fig. 3A)

Matrix remodeling.

Gene expression of *COL1A1* expression increased with PRP 15%, PRP 5% and PRP 1.7% PRP compared to FCS 10% ($p<0.05$), with a peak at PRP 5%, followed by a decrease at PRP 15% (Fig. 3B). Matrix metalloproteinases *MMP1* and *MMP2* followed a similar pattern with the highest expression at PRP 5% and 1.7% but with comparable expressions at FCS 10% (Fig. 3C). Media with all PRP concentrations caused increased expression of *MMP1* and *MMP2* compared to PPP 15% ($p<0.05$). However, the highest expression of *MMP2* was in medium with 10% FCS compared to medium with all concentrations of PRP ($p<0.05$). A strong decrease in gene expression of *MMP2* was observed at PRP 15% compared to FCS 10% ($p<0.05$) and the PRP concentrations 5 and 1.7% ($p<0.05$, Fig. 3C). The gene expression of *COL3A1* was not affected by PRP compared to control conditions FCS 10% and PPP 15% (Fig. 3B).

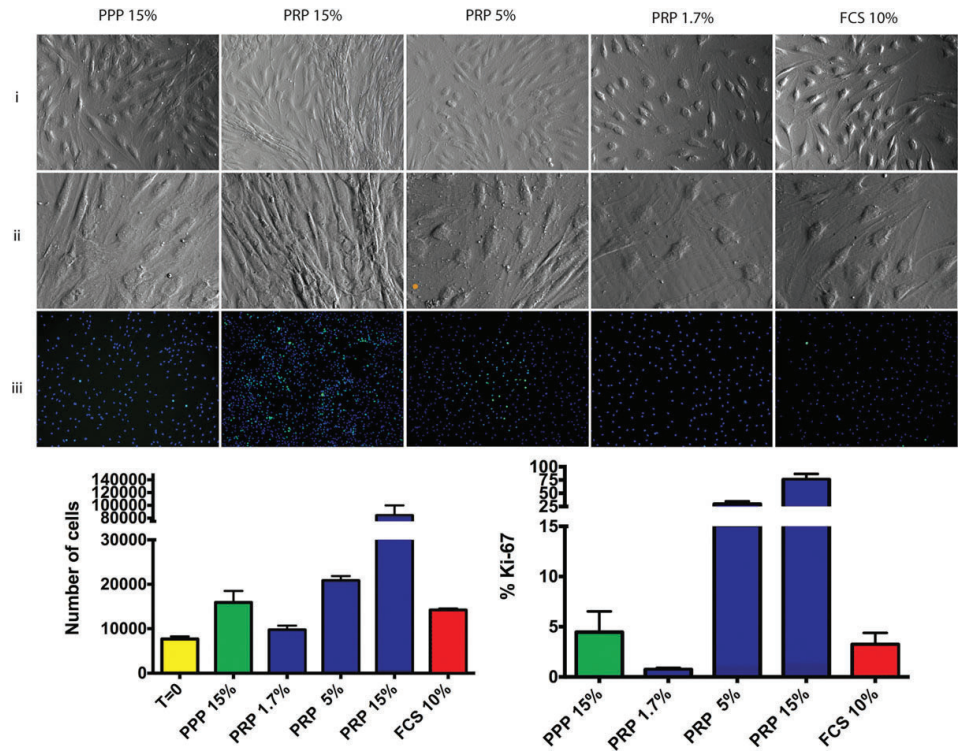

Figure 2 | PRP promotes cell proliferation in a dose-dependent fashion.

Top panel i, ii – light micrographs (10 and 20-fold original magnification respectively) showing the PRP concentration-dependent increased proliferation of ADSC after 3d culture. iii - PRP concentration-increase of proliferation (Ki-67 immunofluorescent staining (green), nuclei (DAPI, blue) of ADSC cultured for 4d.

Lower panel Quantification of actual total numbers of cells per well or fraction of proliferating ADSC (Ki-67) by automated image analyses (Tissue Gnostics Tissue FAXS) corroborates the qualitative observations. Graphs represent triplicates (with SEM) data from n = 3 independent experiments from 3 PRP donors.

Paracrine factors.

The expression of *TGFB1* increased with increasing PRP concentrations, all concentrations induced a higher expression compared to 10% FCS ($p>0.05$, Fig. 3E). Similarly, the expression of *FGF1* followed the concentration of PRP with the highest expression in 15% PRP. Both PRP 15% and 5% increased expression compared to PPP 15% and FCS 10% ($p<0.05$, Fig. 3D). The expression of *IL1B* decreased in a concentration-dependent manner with increasing PRP concentrations in the medium. In medium with 15% PRP the expression of *IL1B* was lower compared to media with FCS 10%, PRP 5% and PRP 1.7% ($p<0.05$, Fig. 3E). The gene expression of *IGF1* followed a similar

pattern, with decreased expression at increasing PRP concentrations. At 15%, PRP affected *IGF1* expression most compared to FCS 10% ($p<0.05$, Fig. 3D).

Angiogenesis

The expression of *VEGFA*, as a marker for pro-angiogenic capacity of ADSC, showed a reciprocal relation with increasing concentrations of PRP in the medium (Fig. 3F). While only 1.7% PRP in medium caused a significant decrease of the expression of *VEGFA* ($p<0.05$). *ANGPT1* expression was also affected; PRP 15% induced a down regulation compared to FCS 10% ($p>0.05$), PRP 5% and PRP 1.7% ($p<0.05$). *ANGPT2* showed a small decrease in the PRP conditions compared to PPP 15% and FCS 10% ($p>0.05$, Fig. 3E).

Dose-dependent influence of PRP on ADSC-induced endothelial sprouting

The addition of 1.7, 5 or 15% PRP to endothelial culture medium instead of FCS resulted in the readily formation of a endothelial sprouting network in all concentrations at 6h which lasted for at least 24h (Supplemental Fig. 4B). The number of loops, branches, and branch lengths peaked at PRP 15% after 6h ($p>0.05$, Fig. 4A). Moreover, networks that had formed in ECM PRP 15% media remained intact for 72h, while in all other conditions the networks had collapsed ($p<0.05$). Much to our surprise, conditioned medium of ADSC that were cultured in the presence of 5% PRP or 15% PRP, strongly inhibited sprouting of HUVEC *in vitro* as observed by the absence of loops or branches (Fig. 4A, Supplemental Fig. 4C). Conditioned medium from ADSC cultured medium with PRP 1.7%, however, induced network formation with comparable number of loops and branches to control media conditioned medium PPP 15%, and conditioned medium FCS 10%. ECM FCS 0% resulted in lowest number of loops and branches ($p>0.05$).

DISCUSSION

Our results show that *in vitro* ADSC respond to Platelet Rich Plasma in a dose-dependent way: PRP caused a dose-dependent increase of the proliferation rate of ADSC, which coincided with increased expression of Ki-67 by ADSC. Moreover, ADSC showed a dose-dependent decrease of several paracrine genes, which is relevant to tissue repair or the take of lipografts. This was corroborated by the near abolished capacity of ADSC to support angiogenic sprouting of endothelial cells *in vitro* after treatment with high concentrations of PRP which to our knowledge, has not yet been demonstrated thus far.

Platelet rich plasma is used as an additive in various clinical indications[20,32], including the use of lipografts. It is generally accepted that the growth factors present in PRP, presumed in high concentration too, stimulate wound healing[33,34], tissue remodeling and revascularization[20] and improve lipograft take[7,15,17]. Clinical evaluation studies on the use of PRP, however, report a large variation of results[7,18,19], which might be due to interindividual variations in the composition of

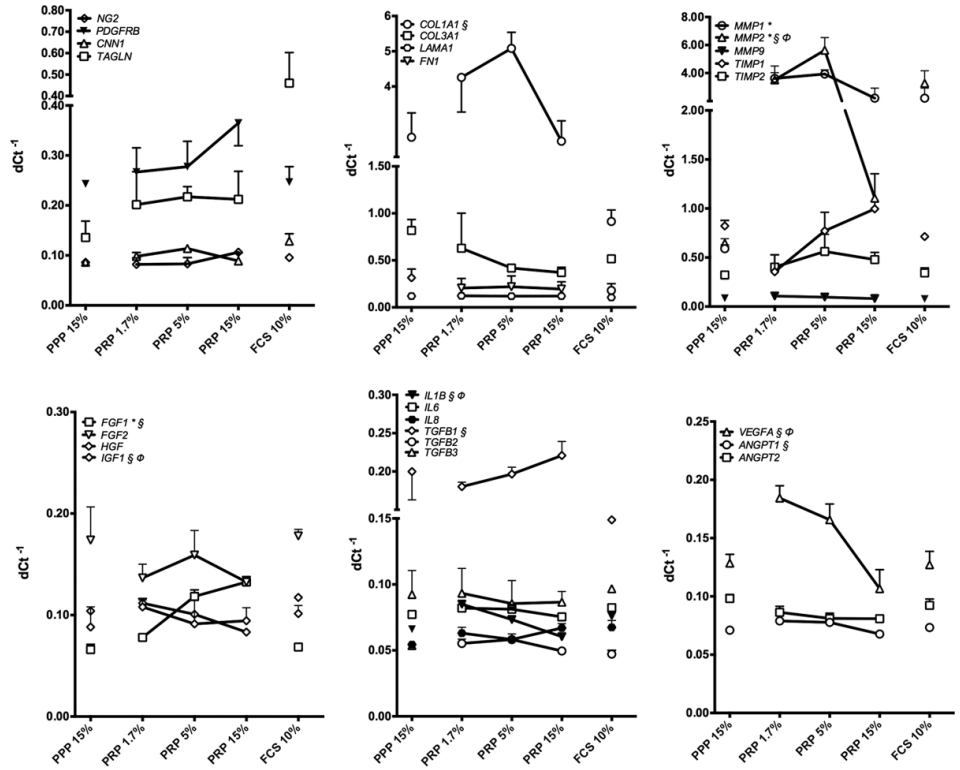

Figure 3 | PRP influences gene expression in ADSC in a dose-dependent fashion. Quantitative RT-PCR analysis of gene transcript levels normalized to ACTB expression. None of the serum or plasma-derived medium additives caused differentiation to smooth muscle-like cells (Top left – TAGLN, CCN1) nor altered the pericytic nature of ADSC (Top left – PDGFRB, NG2). Of all four extracellular matrix (ECM) genes, COL1A1, COL3A1, LAMA1 and FN1, COL1A1 was downregulated in PRP-media compared to FCS controls (Top middle). In contrast, of the ECM remodeling genes, MMP1, MMP2, MMP9, TIMP1 and TIMP2, MMP2 (a gelatinase) were upregulated in high concentration PRP-medium compared to lower concentrations and to PPP as well as FCS controls (Top right). While MMP1 was upregulated compared to both PPP-controls (Top right). Of the pro-mitotic growth factor genes FGF1, FGF2, HGF and IGF1, FGF1 was upregulated compared to both controls, while IGF1 was upregulated compared to FCS controls and showed a PRP-dose-dependent increased expression too (Lower left). Inflammatory genes (IL1B, IL6, IL8, TGFB1, TGFB2 and TGFB3) were not regulated, except for TGFB that was upregulated compared to FCS controls, while IL1B was downregulated in a dose-dependent fashion and compared to FCS controls (Lower middle). The pro-angiogenic genes VEGFA and ANGPT2 were both regulated compared to FCS controls, while VEGFA showed a PRP-dose-dependent downregulation too (Lower right). Graphs represent triplicates (with SEM) data from n = 3 independent experiments from 3 PRP donors. Significant changes in expression ($p<0.05$): * One or more PRP concentration(s) compared to PPP 15%; § One or more PRP concentration(s) compared to FCS 10%; Φ Within PRP concentrations.

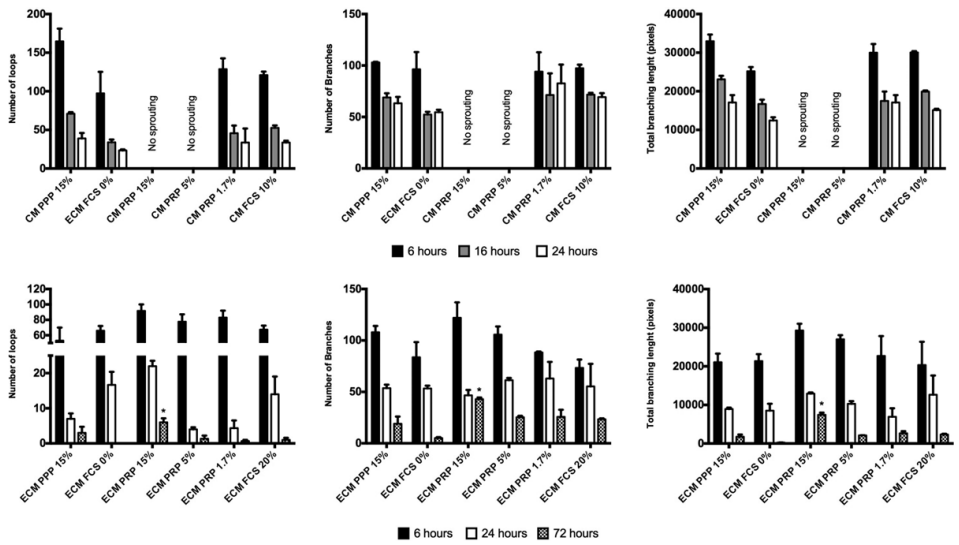

Figure 4A. In vitro sprouting of HUVEC is affected by PRP alone and by PRP-conditioned media from ADSC. Sprouting networks of HUVEC on Matrigel were readily (6h) formed and remained present at 24h, while only in medium with 15% PRP these networks remained stable for 72h (A and online suppl. B and C). Conditioned medium derived from ADSC cultured in media with 5% PRP or 15% PRP abolished the formation of endothelial sprouting networks as compared to controls (online suppl. C). In general, the presence or absence of plasma or serum constituents did not cause differences in the number of loops, branches and total branching length. The number of sprouts, quantified in number of loops and branches as well as their length, calculated by the Angiogenesis-analyzer plugin for Image J. Significant ($p<0.05$): * One or more PRP concentration(s) compared to PPP 15%, FCS 10%. Graphs represent triplicates (with SEM) data from n = 3 independent experiments from 3 PRP donors.

PRP – lipograft mixtures. Different concentrations of PRP have shown to induce varying effects on fibroblast, osteoblasts and endothelial cells[21,22,35-37]. With ADSC playing a key role in fat graft survival and tissue rejuvenation[6,27], the effect of PRP in a possible dose-dependent response on ADSC seems to be crucial. Therefore, our study investigated these presumed effects: a maximum PRP concentration of 15% was chosen because this is the highest clinically feasible concentration available when using disposable PRP-kits, already requiring 4.5mL of PRP in a 25.5mL lipograft.

Our results demonstrated that PRP is a powerful dose-dependent mitogen for ADSC, with two- to fivefold more cells at high PRP concentrations compared to 'normal' serum FCS after 4d culture. This finding fits the results of various other studies on PRP induced proliferation of ADSC[38-40] fibroblasts[22,41] and HUVEC[14,37]. In the study of Kølle *et al.*[6], increasing the number of ADSC in a lipograft had a significant positive effect on graft take, although others have not yet confirmed consensus of this effect. Positive effects on graft survival could be explained by the supportive effect of the ADSC on surrounding cells or due to late differentiation[42] of ADSC into adipocytes.

ADSC cultured in media with PRP maintain their capability to differentiate into adipocytes[43,44], and even potentiate insulin-induced adipogenic differentiation through a serine/threonine kinase Akt-dependent mechanism[45]. Differentiation of ADSC into adipocytes could significantly contribute to end graft volume, as suggested by Kølle *et al.*[6].

In addition we determined that expression of genes related to ADSC function were altered by exposure to PRP, as compared to control media PPP 15% and FCS 10%. Moreover there was a clear influence of different PRP concentrations. These changes found in relative gene expression corroborate data of Amable *et al.*[46], who explored the effects of human platelet lysate on several stem cell types.

Expression of mesenchymal markers was not influenced by PRP, which indicates that in PRP ADSC do not acquire myofibroblast features. This is relevant, because myofibroblasts are related to adverse remodeling and fibrotic tissue processes. However, rather large changes were observed in expression ADSC function related genes. PRP significantly increased expression of genes encoding collagen type I, Matrix metalloproteinases 1 and 2. The increases of these factors indicate that PRP increases capacity of ADSC to facilitate tissue remodelling. Moreover, *TGFB1*, *FGF1* and *IGF1* showed a strong dose dependent upregulation, whereas *IL1B* and *VEGFA* a downregulation. The upregulation of the anti-inflammatory *TGFB1* and simultaneous downregulation of the pro-inflammatory *IL1B* would be beneficial in graft take and wound healing where adverse inflammation could cause graft damage and apoptosis. The upregulation of both strongly mitotic and anti-apoptotic growth factors *FGF1* and *IGF1* would translate to an improved graft take through stimulation of tissue integration and suppression of apoptosis. Together these changes in expression of genes encoding paracrine factors indicate that ADSC switch from a highly pro-angiogenic phenotype with modest matrix remodelling capacities, to phenotype that is not in support of angiogenesis, while tissue remodelling is enhanced as well as proliferation and survival of tissue cells. This, however, remains topic of future research. The changes in gene expression were confirmed by the changes in the effects of conditioned media: ADSC exposed to higher PRP concentrations seem to loose pro-angiogenic properties, as endothelial network formation was blocked by their conditioned media. Possible explanations of this observation can be found in the up regulation of *TGFB1* combined with a decreased expression of *VEGFA*. While both *TGF-β1* and *VEGFA* are associated with angiogenesis by influencing endothelial cells[47,48], changes in their relative availability modify the overall effect. High concentrations of *TGF-β1* in absence of *VEGFA* induce endothelial apoptosis[49], thus blocking network formation.

In contrast to conditioned media derived from ADSC cultured in higher PRP concentrations, direct addition of PRP did not negatively influence endothelial network formation. Moreover, endothelial sprouting was increased by PRP. Increasing PRP concentrations correlated with the formation of more loops and branches, which survived longer as compared to control conditions. PRP effects on endothelial cells are most likely induced by readily available *VEGFA*, *Ang1-Tie2* signaling and

activation of the ERK and phosphatidylinositol-3-kinase–Akt pathways[50,51]. The study of Kakudo *et al.*[14] reported similar positive findings on endothelial network formation, both *in vitro* and *in vivo*, but also showed an inhibitory effect on endothelial network formation at higher PRP concentrations. Our data show that, inhibition at ECM PRP 15% did not occur. With 50µL of media used in our endothelial sprouting assay, compared the 300µL used in the study of Kakudo *et al.*, and differences in cell seeding density ($2x10^4$ cells/well *vs.* $1x10^4$ cells/well in our experiment) the total level of available growth factors may differ significantly, again underlining the importance of the PRP concentration.

PRP, or platelet lysates have several advantages over the use of animal-derived serums in cell cultures[39]. Risk of contamination with animal pathogens and proteins is absent which allows for use in human cell therapy. Although PRP allows for rapid cell expansion in vitro, ADSC gene expression and correlated secretome production changes as compared to FCS[43,46]. The reported therapeutics properties of ADSC cultured on FCS[10,20,52] might inadvertently be lost by cultivation on high platelet lysate or high PRP concentrations, and therefore warrants further study of the altered properties.

Results of this study demonstrate that ADSC respond to Platelet Rich Plasma in a dose-dependent way, and emphasize the need for further research and optimization in the use of PRP as an additive to lipofilling, ADSC enriched lipofilling or ADSC cell base therapies. Dose depended effects on proliferation and ADSC function were found, most likely explaining the variable varying clinical results that have been observed thus far. However, it remains unclear if, and how a higher number of ADSC with altered function induced by PRP may and will contribute to graft survival. Also, in vivo interaction between endothelial cells-ADSC-PRP, and the resulting effect on angiogenesis requires further study.

Furthermore, ADSC cultured on PRP differ from FCS cultured ADSC, losing pro-angiogenic properties. This effect should be taken into account by future studies on ADSC based cell therapies using PRP or platelet lysates as serum.

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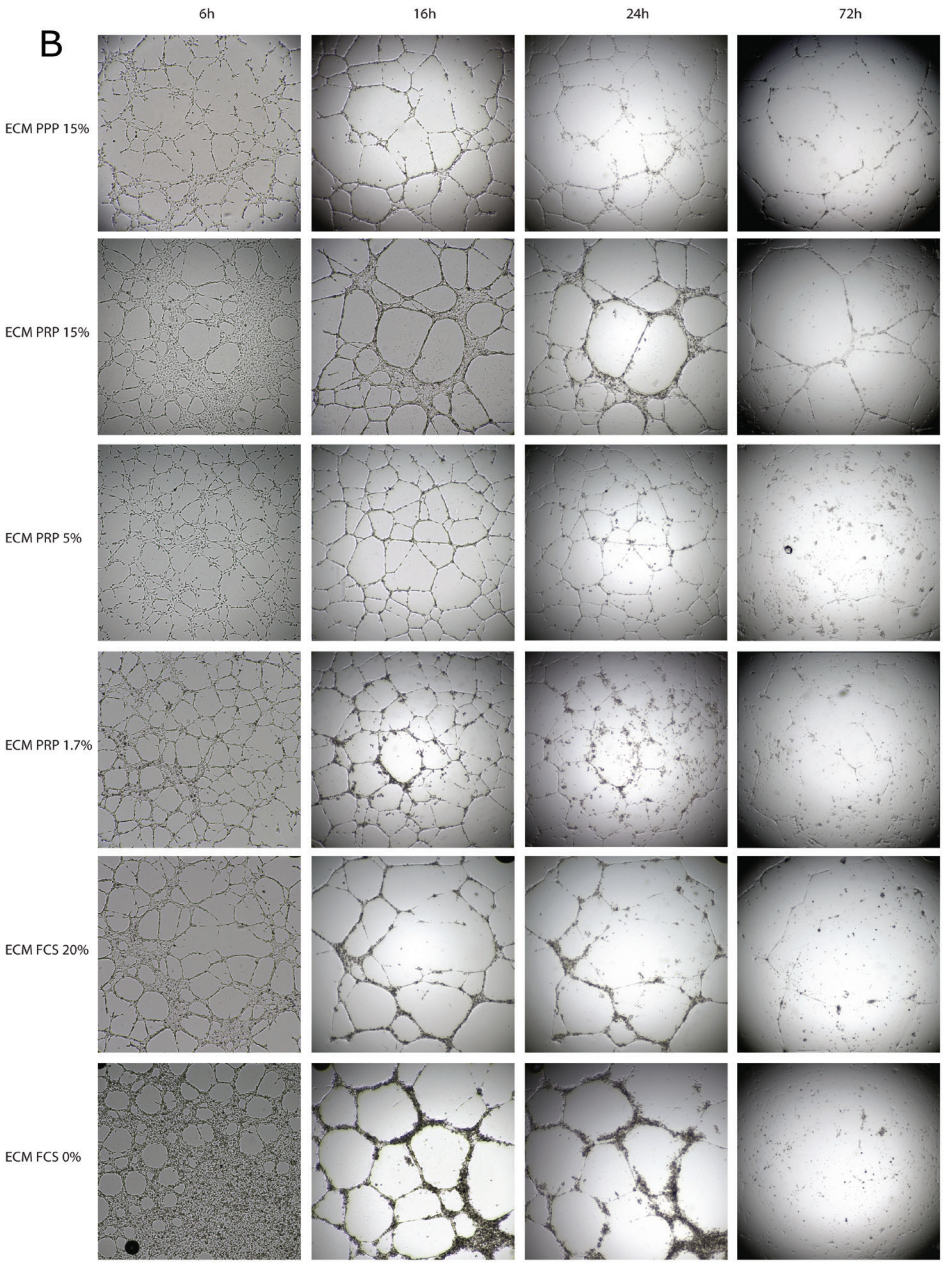
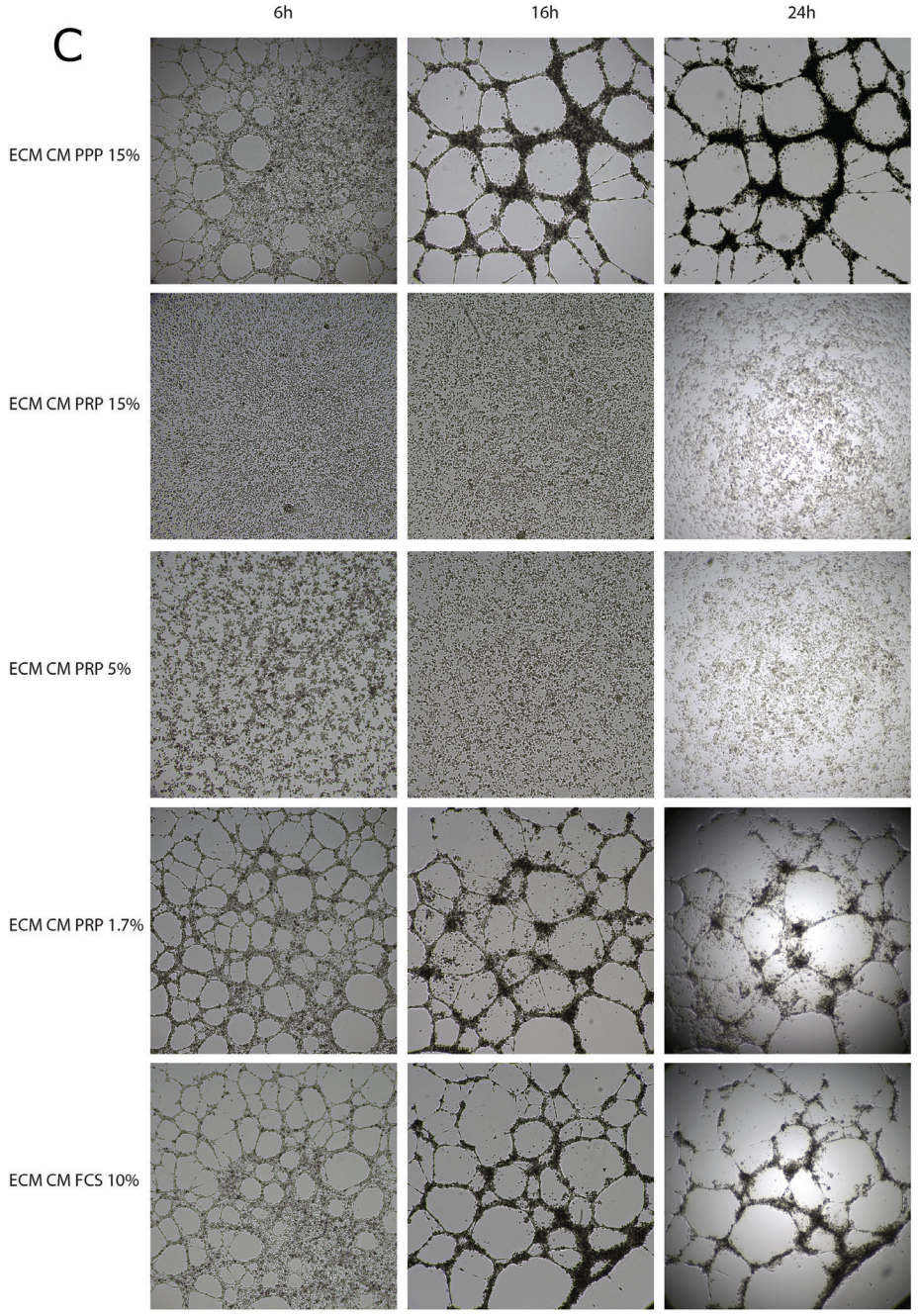
SUPPLEMENTALS

Supplemental figure 4B | Sprouting networks of HUVEC on Matrigel with PRP as serum component.

Supplemental figure 4C | Sprouting networks of HUVEC on Matrigel with conditioned medium derived from ADSC cultured in media with 1.7%, 5% PRP or 15% PRP.



Supplemental figure 4B | Sprouting networks of HUVEC on Matrigel with PRP as serum component. Light micrographs of individual wells of m slide angiogenesis plates coated with Matrigel were taken at 2.5x original magnification.



Supplemental figure 4C | Sprouting networks of HUVEC on Matrigel with conditioned medium derived from ADSC cultured in media with 1.7%, 5% PRP or 15% PRP. Light micrographs of individual wells of m slide angiogenesis plates coated with Matrigel were taken at 2.5x original magnification.